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LIGHT-MICROSCOPIC AND MORPHOMETRIC PROPERTIES OF ARGYROPHILIC NUCLEOLAR ORGANIZING REGIONS IN DEEP EPIDERMAL RIDGES OF HUMAN THICK SKIN

Aleksandar Petrović[1], Vladimir Petrović[1], Dragan Jovanović[2], Aleksandra Antović[3], Miroslav Milić[3], Hristina Kocić[2]

The epidermis of the thick skin on the flexor sides of hands and feet has a very complex superficial relief, known as dermatoglyphics, constantly maintained by precise, spatially coordinated regeneration and differentiation, whose potential lies within the deeper epidermal parts – rete ridges. One of the proliferative markers, AgNORs, represents neucleolar organizing regions, that after histochemical staining with silver ions can be observed as black dots in the nucleus. The aim of this study was to estimate morphometric properties of AgNORs in different micro-topographical compartments of thick skin epidermis, such as deep intermediate and limiting epidermal ridges. Necropsy samples of thick skin were taken from the tips of big toes of fifteen cadavers, and routinely processed to paraffinized microtome sections, which were stained with hematoxylin-eosin, and silver-based method for staining nucleolar organizing regions. Morphometric analysis was performed separately on basal keratinocytes of intermediate and limiting epidermal ridges. Suprabasal layer of tips, as well as basal layer of intermediate ridge sides, as a sign of higher proliferative status, showed a higher number of silver-stained nucleolar organizer regions with small average values of the area. According to AgNORs morphology, proliferation was sporadically and diffusely present in basal, as well as in suprabasal layer of tips, and sides of limiting ridges.

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[1]University of Nis, Faculty of Medicine, Department of Histology and Embryology, Niš, Serbia
[2]University of Nis, Faculty of Medicine, Department of Dermato-Venereology, Niš, Serbia
[3]University of Nis, Faculty of Medicine, Department of Forensic Medicine, Niš, Serbia

Contact: Aleksandar Petrović
Blvd. dr Zorana Djindjica 81, 18000 Niš, Srbija
E-mail: aleksandar.petrovic@medfak.ni.ac.rs

Introduction

Glabrous skin of palms, soles, and flexor sides of fingers and toes, exposed to constant mechanical stimulation, has evolutionary considerably thicker epidermis than the skin on other anatomical integument regions (1,2). In these topo-anatomical regions, the epidermis is characterised by its prominent height and specific superficial relief composed of parallel linear ridges, and sulci between them, in the form of whirls, loops, and arches, making individually specific patterns, named dermatoglyphs. Their configuration is genetically determined and absolutely individual (3,4). The deep part of epidermis follows a diversified structure of the surface and forms deep epidermal rete ridges:

1) intermediate, which are positioned under superficial ridges,
2) limiting, located under superficial sulci, and
3) transverse, which intermittently bridge the previous two ridge types (5–9).

AgNORs (Argyrophilic Nucleolar Organizer Regions) have been defined as a set of proteins associated with ribosomal genes rDNA of nucleolar organizer regions, showing a selective affinity to bind silver ions (10). In cellular biology, the nucleolar organizer regions (NORs) are located on the secondary constrictions of acrocentric human chromosomes (13^{th}, 14^{th}, 15^{th}, 21^{st}, 22^{nd}) and contain rDNA. During interphase, these genes are located in nucleoli, with associated proteins of ribosomal subunits biogenesis. The major proteins involved in rRNA transcription and processing are RNA polymerase I, upstream binding factor (UBF), DNA topoisomerase I, nucleolin or C23 protein, fibrillarin and numatrin or B23 protein. In interphase, the NORs are located in the fibrillar centre, made of rDNA loops involved in rRNA transcription (except for 5S rRNA), and of NOR proteins (RNA pol-1, DNA topoisomerase-1, B23,

C23 and fibrillarin) (10). Among the argyrophilic nucleolar proteins, nucleolin and protein B23 were in normal proliferating cells estimated to account for 60–75% of the global AgNOR staining (11, 12). All of these proteins possess a great affinity toward silver ions, and argyrophila ultrastructurally extends from the fibrillar centre to dense fibrillar component (13–15). Upon staining with silver ions, NORs become apparent as black dots located inside the nucleolar region. The degree of proliferation in one cellular population could be evidenced by the detection of morphologic changes in nucleoli during cellular divisions. During interphase, the argyrophilic reaction toward AgNOR proteins is visible as black dots clustered in nucleoli. In the prophase of cell division, the AgNORs of nucleoli disassociate, and are shortly visible as distanced small black dots, which disap-pear during the rest of the division, reappearing at a reverse reassembly during the telophase, followed by formation of functional nucleoli (11, 16–19).

Epidermal regeneration is based on basal stratum stem cells, which by rare asymmetric divisions are self-maintaned and produce transit-amplifying cells, where the latter, in 3-5 further cell divisions, give rise to postmitotic keratinocytes that differentiate toward the corneal stratum (20–30). In the available literature only a few articles reported about the proliferative activity of deep epidermal ridges of the human thick skin by using different methods: [^{3}H] thymidine,(31) cell cycle S phase labeling with deoxyoligonucleotide probes to histone mRNAs, (30) and analyzing distribution of Ki67 positive nuclei in epidermal basal and suprabasal layers adjacent to acral and nonacral human nevi (32).

Although many papers, reporting about AgNOR expression in different epidermal lesions also presented the values for normal epidermis thin skin, the available literature lacks the data about proliferative activity of human thick skin epidermis by using the AgNOR staining methodology (33–36). The aim of this study was to quantify the number and area of AgNORs in nuclei of thick skin basal and suprabasal keratinocytes, in the regions of intermediate and limiting deep epidermal ridges.

Material and methods

This research was conducted at the Department of Histology and Embryology and Department of Forensic Medicine (Faculty of Medicine, University of Nis). The material consisted of skin samples taken from the tips of big toes from 15 cadavers of male gender, with age range from 35 to 52 years. The necropsies were excised perpendicularly to the skin surface, and transversally to the longer axis of superficial ridges, obtaining tissue samples of about 7x4x4 mm. The material was fixed in aqueous 4% formaldehyde solution, and routinely processed to 4 μm thick paraffinized tissue sections. The tissue slides were stained routinely with hematoxylin and eosin, and histochemically for AgNOR as recommended by the International Committee on AgNOR Quantitation – ICAQ (17). The research was carried out in compliance with the legal regulations and ethical standards for retrospective studies, which was approved by the local Ethics Committee.

A short description of AgNOR staining method by ICAQ

Tissue slides were deparaffined, rehydrated through decreasing concentrations of aqueous solution of ethanol and were brought to distilled water. Just before the staining, a developer for staining was made of 0,6% gelatin dissolved in deionised water, to which formic acid was added in order to obtain a 0,33% solution. The developer was warmed up to 37^{O}C before the addition of silver-nitrate, to make the final 33% staining solution, in which microscopic slides were immersed. The staining was performed in a dark chamber, incubation was carried out at 37^{O}C for 13 minutes. In continuation of the staining process, the stain was poured out, slides were washed several timed in deionised water, fixated in 5% sodium-thiosulphate, dehydrated, and mounted in glycerine.

Microscopic slides were analysed on a light microscope (Olympus BX50, Japan) equipped with Leica DMR digital camera (Leica Micro-Systems, DFC 295). From each of the examined tissue sample, for the purpose of morphometric AgNOR analysis, with the use of immersion oil, digital micro-photographs of minimally 5 pairs of deep epidermal ridges (intermediate and limiting) were taken under x2000 magnification. Morphometric analysis of the number and transectional surface area of AgNORs was done by interactive separation of AgNORs in Olympus Micro-Image Software, v. 4.0 for Windows (Media Cybernetics, Silver Spring, USA). Statistical analysis was done using the Jandel Sigma Stat 2.0 (SPSS Inc., Chicago, USA) software, using its functions for descriptive and comparative statistics of Mann-Whitney test. The differences between the values were considered statistically significantly different for $p < 0.05$.

Results

Microscopic slides of thick skin, stained with hematoxylin-eosin, displayed normal morphology characterized by well developed epidermis, which showed on its dermal side repeating and alternating, narrower intermediate and wider limiting ridges (Figure 1).

Those two types of deep epidermal ridges are different not only by their location and general morphology, but also by specific cellular composition, and their distribution. The basal layer of intermediate ridge is composed of small cubical cells with scarce basophilic cytoplasm, one centrally positioned rounded or slightly oval nucleus, and one variably apparent nucleolus. The basal cells of limiting ridge are elongated, almost prismatic and have more developed acidophilic cytoplasm. One larger oval euchromatic nucleus is located in the apical part of these cells, containing one or two well developed nucleoli.

On thick skin samples stained with ICAQ methodology, a lesser number of larger AgNORs could

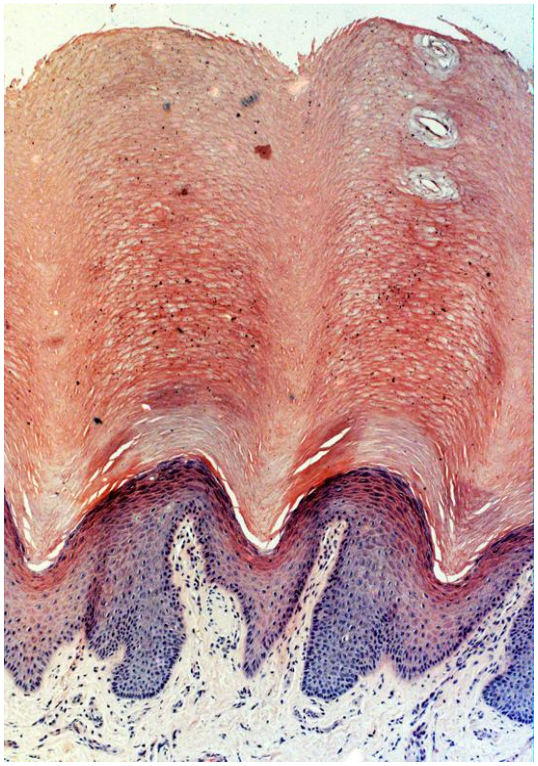

Figure 1. Histology of the thick skin epidermis. Deep intermediate ridges positioned under superficial ridges (on the right, spiral lumen of acrosyringia), and limiting under superficial sulci (in the middle) (hematoxylin-eosin, x100).

be observed in the nuclei of intermediate ridge tips basal layer. The intermediate ridge suprabasal layer had slightly larger nuclei with noticeably more numerous AgNOR structures and smaller average surface area per object (Figure 2a). The number of AgNORs and their areas showed a high statistical difference between the basal and suprabasal layer of intermediate ridge tips ($p < 0,001$) (Tables 1 and 2). On the sides of intermediate ridges, basal layer AgNORs were more numerous compared to suprabasal layer ($p < 0,05$), while smaller average values of their transectional area showed no statistically significant differences (Figure 2b, Table 1 and 2).

Within the tips of limiting ridges, a lower number of basal layer cells displayed "disassembling" nucleoli on separate, smaller components, observable as AgNOR single positive dots or partially grouped dots. However, single nucleoli could be observed in the majority of nuclei. In this region, measured AgNORs number and area values did not show statistically significant differences between the basal and suprabasal layer ($p > 0,05$) (Figure 2c, Table 1 and 2). The nuclei of basal layer in limiting ridge sides show lower values of AgNOR area compared to nuclei of suprabasal layer, with a statistically significant difference ($p < 0,05$). AgNOR structures were more numerous in basal than in suprabasal layer, however without a statistical significance ($p > 0,05$) (Figure 2d, Table 1 and 2). The higher parts of spinous stratum showed single nuclei clustered AgNOR pattern.

Discussion

Interpretation of AgNOR distribution pattern as a manifestation of cell cycle phase was mainly defined in the previous studies for the neoplastic cells, and mostly in the light of its prognostic or predictive significance. Combined MIB-1 and AgNOR staining and cytometry of cancer cells have been used to show that cell cycle time and the size of the ribogenesis machinery are co-regulated (10, 37). However, Ag-NOR distribution interpretations done by different authors for the same tumor types are rarely comparable. A definitive standardization of AgNOR staining and quantification has not yet been achieved, and this could be related to the use:

1) of different silver-staining methods for NOR labeling; and

2) variety of procedures for AgNOR proteins quantification (16).

Although many molecular studies reported the association of argyirophilic nucleolar proteins with cell cycle, it is interesting that the relation between morphology/pattern of AgNORs expression and life cycle of normal cells was almost a neglected question. A number of studies carried out in different tumour types have demonstrated that malignant cells frequently present a greater content of AgNOR proteins than the corresponding non-malignant cells (10). In cancer cells, AgNOR proteins expression was seen as strictly related to cell duplication rate, and there was a general consensus that AgNOR size or number was related to proliferative activity – the larger the AgNORs, the shorter the population doubling time (10, 37).

However, the interpretation of AgNOR expression pattern in normal cells should be considered quite differently than in malignant cells. Namely, the expression of AgNOR number and quantity of argyrophilic proteins are different for various normal diploid cellular phenotypes, and it seems that further standardized quantification should be implemented. The most important event to be taken in consideration when aiming at AgNOR distribution interpretation in normal cellular phenotypes is their nucleolar disassembly during prophase and their reassembly during telophase. During that process, NORs of nucleoli physically separate and reduce their volume, become invisible during the rest of cell division, and reappear and reassemble at telophase in the process of nucleologenesis, continuing with the onset of rRNA synthesis at the NORs (38–41). The main AgNOR proteins detected during active transcription and proliferation are nucleolin and protein B23. The amount of these proteins rises during S phase, and accumulates maximally at G2 phase, being 1.5 times more abundant compared to G1. During interphase, RNA polymerase I subunits, proportionally to their lower presence compared to nucleolin and B23, take a lesser part in general AgNOR staining. On the contrary, during mitosis, AgNOR staining of mitotic NORs reveals the presence of RNA polymerase I

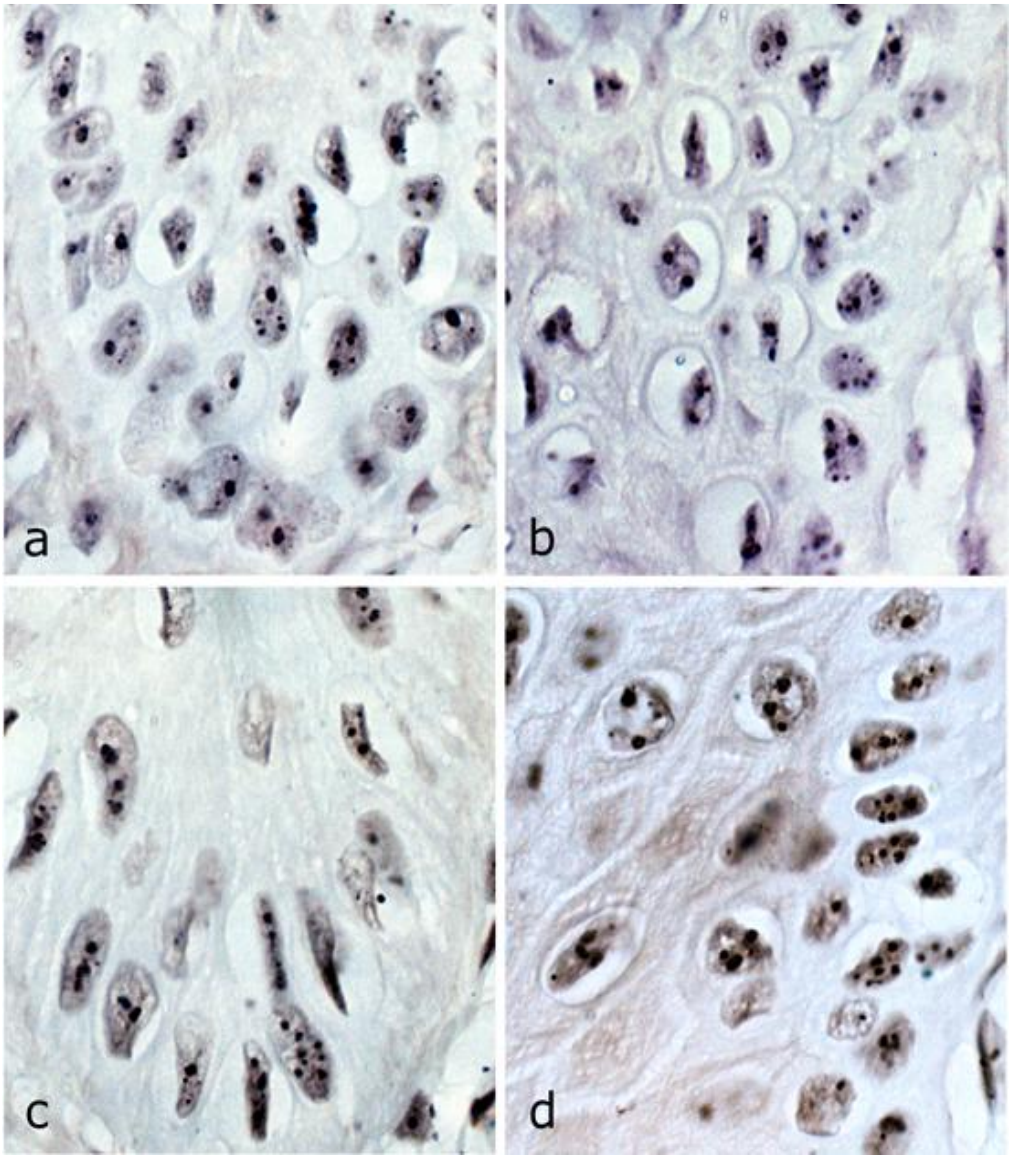

Figure 2. Morphology of AgNORs in the nuclei of thick skin epidermal keratinocytes by different microtopographical regions/compartments: a) tip of intermediate ridge, b) side of intermediate ridge, c) tip of limiting ridge, d) side of limiting ridge (ICAQ – AgNOR, x2000, immersion).

Table 1. Average values of surface area (μm^2) and their standard deviations (X ± SD) of AgNORs in tips and side of intermediate and limiting ridges. (n = 15)

Part of ridge	IB	IS	LB	LS
	(P value)		(P value)	
Tip of ridges	1.72 ± 0.46	0.38 ± 0.17	1.17 ±1.04	1.98 ± 0.75
	(< 0.001)		(>0.05)	
Ridge sides	0.27 ± 0.07	0.50 ± 0.26	0.80 ± 0.34	1.55 ± 0.25
	(> 0.05)		(< 0.05)	

IB – basal layer of intermediate ridge, IS – suprabasal layer of intermediate ridge, LB – basal layer of limiting ridge, LS – suprabasal layer of intermediate ridge; * statistical differences were tested by Mann-Whitney test

Table 2. Average number of AgNORs and their standard deviations (X ± SD) per nuclei of keratinocytes in tips and sides of intermediate and limiting ridges. (n = 15)

Part of ridge	IB	IS	LB	LS
	(P value)		(P value)	
Tip of ridge	1.15 ± 0.37	4.6 ± 1.31	2.07 ±1.06	1.88 ± 1.25
	(< 0.001)		(>0.05)	
Ridge sides	5.50 ± 1.30	3.67 ± 0.82	2.17 ± 2.40	1.50 ± 0.58
	(< 0.05)		(> 0.05)	

IB – basal layer of intermediate ridge, IS – suprabasal layer of intermediate ridge, LB – basal layer of limiting ridge, LS – suprabasal layer of intermediate ridge; * statistical differences were tested by Mann-Whitney test

subunits and upstream binding factor (UBF) which are the proteins of the transcription machinery. Therefore, the test for human cancer cell proliferation is mostly based on the level of nucleolar proteins that are not directly involved in rDNA transcription (11).

Examining epidermal differentiation in cynomoglus monkies and humans, Lavker and Sun noticed that palmar epidermis has two morphologically different, spatially segregated populations of basal keratinocytes (31). According to ultrastructural characteristics and kinetics of the cell cycle, the mentioned authors hypothesized that low proliferative basal layer keratinocytes of intermediate ridge tips were primitive differentiated cells, closer to stem cells, while serrated keratinocytes of limiting ridges, because of specifically differentiated cellular protrusions on basal pole and cytoplasmatic presence of tonofilaments, were considered as cellular population involved in augmentation of epidermal-dermal contact surface.

The differences noticed in AgNOR distribution, between intermediate and limiting ridges could be influenced by ridge morphology, cellular differentiation (31, 42, 43) and by specific local demands toward germinative compartment (31, 44–46). More numerous and smaller AgNORs per nucleus, evidenced in our research, primarily in the suprabasal layer of intermediate ridge tips and basal layer of its flanks, may be interpreted as the presence of expected and more intense proliferative activity. Larger and single nucleoli in the nuclei of intermediate tips basal layer cells, indicate the population of cells detoured form proliferation activities, and their statistically different number of AgNORs and average area, compared to the nuclei of suprabasal layer, suggest a model of quick and rare divisions within basal layer and switching proliferation and regenerative activity in the population of suprabasal layer. Non-proliferative nature of the intermediate ridge tips basal layer may be partially explained by the "dilution" of keratinocyte population by the presence of Merkel cell complexes, melanocytes, and acrosyringia. The basal layer of intermediate ridge sides / flanks, which shows a statistically significant difference of AgNOR structure area, does not show a statistical difference in their number when compared to suprabasal region, although it contains more AgNORs. Such a finding implies intensive proliferation, which continues through suprabasal compartment, giving "power" to "stream of keratinocytes" in the region of intermediate ridge, supposedly for the purpose of protruded surface ridge formation, by generation of larger number of newly produced, and latter through the rest of commited compartment, terminally differentiated keratinocytes. A higher distribution of proliferating cells, as seen by AgNOR interpretation, located in the germinative compartment of the intermediate ridge sides could represent a larger transit-amplifying compartment, based on a steeper basement membrane orientation of the ridge side (25, 47).

The contrast between the presence of epidermal suprabasal proliferation (32, 45, 46), and its insignificant appearance in higher layers (24, 48) was also reflected in our material as a pattern of AgNOR expression in the form of single nucleoli cluster in the cells of the higher parts of spinous stratum. The regulation of transit-amplifying compartment above the basal layer (49–51), and its specificity compared to upward terminally differentiating compartment, (24, 48) has a significance in the pathogenesis of epidermal hyperproliferative diseases, i.e. in psoriasis (52–57).

In the limiting ridge, basal and suprabasal layer AgNOR distribution pattern differed from the one evidenced in the intermediate ridge. The cells of the limiting ridge basal and suprabasal layer did not differ significantly in the number of AgNORs per nucleus, and area of AgNORs in tips of limiting ridge basal layer had smaller values compared to suprabasal layer, however without a statistically significant difference, leaving the impression of diffuse proliferation equally distributed within both of these layers. If we take into consideration the higher value of standard deviation in basal layer, it could be assumed that statistically significant difference was absent due to mixed population of cells with proliferative and cells with resting (interphase) morphology. Generally, the limiting ridges, as in the results of Lavker and Sun (31) that had more of an anchoring role toward papillary dermis and formation of superficial sulci, expectedly should have had a more tardive proliferative activity, the property which was almost equally distributed throughout the whole length of basal and suprabasal layer.

The number and area of transsectioned AgNORs, given our results, are generally in the va-

lue range reported in the available literature for human thin skin epidermis (33, 34, 36). However, these data are not directly comparable, due to specificities of thick skin epidermis, as well as because we focused on the germinative compartment and its components, the basal layer and suprabasal layer of spinous stratum. Heinisch et al. have reported the values for normal basal layer of human epidermis, where the AgNOR number was 3.3 ± 0.5, and AgNOR area 1.76 ± 0.21 μm^2. The mentioned findings are similar to our results, representing average values for whole length of examined basal layer, with the difference that our results are divided in four different topographic locations, within epidermal intermediate and limiting ridges.

It is much more likely that obvious morphological difference between intermediate and limiting ridges, followed by a specific expression pattern of various molecular indicators of cell cycle and differentiation, strengthen the impression of two separate tissue differentiation systems which again function in equilibrium to maintain epidermal homeostasis. In addition, the spinous strata cell morphology and different keratin types expression, separately specific for intermediate and limiting ridges (43,58), could be the evidence of different keratinocyte lines, originating from two stem cell precursors (59). Moreover, genetical labeling has proven that normal epidermal proliferating units are spatially organized without regularity of segregation or lateral migration of labeled cells (47).

Conclusion

Intermediate and limiting deep epidermal ridges express two different patterns of proliferation, recorded by silver labeled nucleolar organizer regions. According to AgNORs, higher proliferative activity in deep intermediate ridges is present in the suprabasal layer of tips and basal layer of the ridge flanks, while the proliferation is sporadically and diffusely distributed in tips and flanks of limiting ridges basal and suprabasal layers.

The thick skin represents a suitable research model of epidermal organization, necessary for understanding not only the complexities of regional histo-architectonics but also general mechanisms of tissue homeostasis and cell cycle morphology.

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SVETLOSNO-MIKROSKOPSKE I MORFOMETRIJSKE KARAKTERISTIKE ARGIROFILNIH NUKLEOLARNIH ORGANIZACIONIH REGIONA U DUBOKIM GREBENIMA EPIDERMA DEBELE KOŽE ČOVEKA

Aleksandar Petrović[1], Vladimir Petrović[1], Dragan Jovanović[2], Aleksandra Antović[3], Miroslav Milić[3], Hristina Kocić[2]

[1]Univerzitet u Nišu, Medicinski fakultet, Katedra za histologiju i embriologiju, Niš, Srbija
[2]Univerzitet u Nišu, Medicinski fakultet, Katedra za dermatovenerologiju, Srbija
[3]Univerzitet u Nišu, Medicinski fakultet, Katedra za sudsku medicinu, Niš, Srbija

Kontakt: Aleksandar Petrović
Bul. Dr Zoran Đinđić 81, Niš, Srbija
E-mail: aleksandar.petrovic@medfak.ni.ac.rs

Epiderm debele kože fleksornih strana šaka i stopala svoj složeni površinski reljef, dermatoglife, održava konstantnom i prostorno-koordinisanom regeneracijom, čije rezerve su smeštene unutar njegovih dubokih grebena. Jedan od proliferativnih markera, AgNOR, predstavlja nukleolarne organizacione regione (NOR), koji se po histohemijskom obeležavanju jonima srebra (Ag) vide kao crne tačke unutar nuklearnog područja. Cilj ove studije bio je utvrđivanje morfometrijskih osobina AgNOR u dubokim intermedijarnim i limitantnim grebenima epiderma debele kože. Uzorci debele kože jagodica palaca stopala petnaest leševa rutinski su obrađeni do parafinizovanih mikrotomskih tkivnih isečaka, a zatim obojeni hematoksilin-eozinom i histohemijskom metodom za obeležavanje nukleolarnih organizacionih regiona jonima srebra. Morfometrijska analiza je izvedena odvojeno na bazalnim i suprabazalnim keratinocitima intermedijarnih i limitantnih grebena. Suprabazalni sloj vrhova, kao i bazalni sloj strana intermedijarnih grebena, kao znak višeg deobnog stanja, pokazali su veći prosečni broj AgNOR, male površine preseka. Prema morfologiji AgNOR, proliferacija je sporadično i difuzno zastupljena, kako u bazalnom tako i u suprabazalnom sloju vrhova i strana limitantnih grebena.

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Ključne reči: *bojenje srebrovim solima, organizacioni regioni jedaraca, epiderm, kompjuterizovana analiza slike*